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THE ROUTINE CULTIVATION OF TUBERCLE BACILLI FROM THE SPUTUM BY PETROFF'S METHOD

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A method for cultivating the tubercle bacillus from discharges, excreta, and tissues which would give a large percentage of positive results in positive cases and would not consume too much time would be of inestimable value. It would open up new avenues for study and classification of the tubercle bacillus, for finding its virulence (as derived from various sources) and probably also for study of the immunologic phases of tuberculosis. A method recently described by Petroff[1] offered many advantages over previous methods for the isolation of the tubercle bacillus, and seemed worthy of an exhaustive trial especially as a routine procedure. The method consists briefly of two steps: The destruction of many of the rapidly growing micro-organisms by means of sodium hydroxid, and the inoculation of the resultant material on an egg medium similar to the one introduced by Dorset, but containing gentian violet in concentration of 1 : 10,000 to inhibit the growth of the contaminating organisms which were not destroyed by the sodium hydroxid. The preparation of the medium consisting of whole egg, beef or veal juice and gentian violet was described by Petroff as follows:

Meat Juice: 500 gm. beef or veal are infused in 500 c c of a 15% solution of glycerol in water. Twenty-four hours later the meat is squeezed in a sterile meat press and collected in a sterile beaker.

Eggs: Sterilize the shells of the eggs by immersion for 10 minutes in 70% alcohol or by pouring hot water on them. Break the eggs into a sterile beaker and after mixing the eggs well, filter through sterile gauze. Add 1 part by volume meat juice.

Gentian Violet: Add sufficient 1% alcoholic gentian violet to make a dilution of 1 to 10,000. Tube about 3 c c in each sterile test tube and inspissate for 3 successive days; on the 1st day at 85 C., until all the medium is solidified, changing the places of the tubes if necessary; on the 2nd and 3rd days for more than 1 hour at 75 C.

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[1] Jour. Exper. Med., 1915, 21, p. 38.

[2] Am. Med., 1902, 3, p. 555.

Fresh sputum is advisable. Equal parts of sputum (about 5 c c) and 3% sodium hydrate are well shaken and left in the incubator for 20-30 minutes until the sputum is fairly well digested. The sputum is then neutralized to sterile litmus paper with normal hydrochloric acid, centrifugalized and the sediment inoculated into several test tubes containing the mediums described in the foregoing. Neutralization is not necessary but is advisable.

Petroff obtained 69 positive cultures from 69 specimens of sputum, 6 of those being negative by direct microscopic examination, but having been positive for tubercle bacilli at some time. In a final report on the use of this method he[3] was able to isolate and cultivate the tubercle bacillus from 129 sputums of 135 specimens. In a brief article on the use of the Petroff method Mitchell and Simmons[4] report that good growth was obtained from 28 of 35 specimens of sputum, all positive by smear, while 6 were contaminated and 1 revealed no growth.

In a preliminary report on the use of Petroff's method of isolating the tubercle bacillus, Keilty[5] calls attention to the importance of neutralization after the use of the sodium hydroxid, avoiding drying of the culture and keeping the incubator temperature between 37 and 38 C. and finally reports[6] having examined 25 sputums of which 18 were positive by smear, 12 of which gave growth; of the 7 negative by smear 1 showed growth. Of the 12 positive cases, 4 were obtained in pure culture and 8 showed microscopic evidence of growth (being overgrown by contamination later). Of the 4 pure cultures, 2 averaged 10-14 organisms to a field on the original sputum examination and 2 showed 1 to several fields. Of the 18 positive sputums, only 4 had many organisms to a field and 2 of these gave pure cultures while 1 had microscopic evidence in culture. Of the 4 pure cultures, 1 case showed 2 contaminations of 4 tubes inoculated. The other 3 grew without contamination. The first appearance of gross growth averaged 14-21 days. Soparkar[7] was able to isolate tubercle bacilli from sputum that was 20 days old by the Petroff method and found that even 2½% sodium hydroxid at 37 C. up to 3 hours did not kill tubercle bacilli. He gave no figures on his number of positive findings, but reports he was able to grow the bacilli by this method even when animal inoculation and the microscope failed to find them. Stewart[8] used the original Petroff method on 37 specimens of sputum positive by microscopic examination and obtained growth from 24, or 64.8%. The shortest time required for growth was 2 weeks (in comparison to 7-14 days observed by Petroff) while the average period was 3 to 4 weeks and in some instances colonies could not be detected in less than 6 weeks after inoculation. The organism was recovered in 69.2% of samples of milk (13 samples were infected, 4 with human [all gave growth] and 9 with bovine [5 gave growth]) that were artificially infected.

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[3] Petroff, S. A.: Bull. Johns Hopkins Hosp., 1915, 26, p. 276.

[4] Jour. Am. Med. Assn., 1915, 65, p. 245.

[5] Jour. Exper. Med., 1915, 22, p. 612.

[6] Ibid., 1916, p. 41.

[7] Indian Jour. Med. Research, 1916, 4, p. 28.

[8] Jour. Exper. Med., 1917, 26, p. 755.

The investigations in this paper were made with a twofold purpose, to obtain cultures of tubercle bacilli for the study of their virulence and to gain information on the various steps involved in the technic of cultivating tubercle bacilli according to the method devised by Petroff. In order to determine whether the acidity of the medium is of any practical significance in determining the growth of tubercle bacilli, as Petroff and Dorset[9] both paid no particular attention to the reaction of the egg medium, the bacilli were grown on 5% glycerol agar of varying acidity with the results indicated in Table 1.

TABLE 1

GROWTH OF TUBERCLE BACILLI ON GLYCEROL AGAR OF DIFFERENT REACTIONS

Reaction*	Alkaline			Neutral	Acid			
	2.0	1.0	0.5		0.5	1.0	2.0	4.0
No. 1305—Recently isolated virulent human bacillus	−	+++	+++	+++	+++	+++	−	−
Bovine—Old laboratory strain	−	++	+++	+++	+++	+++	−	−

The results given are the readings made after 1 month incubation at 37 C. averaging 4 tubes in each case.

* The reaction given is in the terms of normal alkali or acid using phenolphthalein as indicator.

It is to be noted that on 5% glycerol agar a good growth of tubercle bacilli is obtainable within wide range (from 1.0+ to 1.0—) of reaction of the medium. Szaboky[10] found the range of reaction on egg mediums also to have wide limits, the best growth occurring on strongly acid mediums, but strongly alkaline reaction or neutrality not preventing fairly good growth.

Likewise the range of use of glycerol was wide as was indicated by the original investigations of Nocard and Roux[11] and also by the following experiment: A series of tubes of Dorset egg medium were prepared containing none and varying amounts of glycerol and these were then inoculated with recently isolated virulent human tubercle bacilli and an old laboratory bovine strain (Table 2).

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[9] Am. Med., 1902, 3, p. 555.

[10] Centralbl. f. Bakteriol., 1907, I, O., 43, p. 651.

[11] Ann. de l'Inst. Pasteur, 1887, I, p. 19, and Centralbl. f. Bakteriol., 1887, 2, p. 136.

From these preliminary experiments it is to be noted that the glycerol content or the acidity of the mediums within wide ranges (1.0+ to 1.0—) play no important part in the cultivation of tubercle bacilli.

Petroff in his first contribution lays great stress on the use of meat juice—beef or veal—and on inspissation in the preparation of the egg medium. In order to determine whether ordinary bouillon containing beef extract and peptone could not be substituted for the meat juice and whether higher temperatures, as for instance autoclaving instead of inspissating the medium, would not answer the purpose the following experiment was made.

TABLE 2

EFFECT OF DIFFERENT GLYCEROL CONTENT ON THE GROWTH OF TUBERCLE BACILLI ON DORSET'S EGG MEDIUM

	Glycerol per cent				
	0	1	2	4	6
1769*	—	++	++	++	++
1907	—	++	++	++	+
461	—	++	++	++	+
1584	—	++	++	++	++
1885	—	++	++	++	++
924	—	++	++	++	+
1769	—	++	++	++	++
Bovine†	—	++	++	++	++

* The numbers indicate recently isolated human tubercle bacilli.

† An old laboratory bovine strain obtained from Dr. Paul A. Lewis and accustomed to growing on glycerol agar.

The results are the readings of 2 tubes inoculated, observations made 1 month after cultivation at 37 C.

Four culture tubes of gentian violet egg medium were inoculated with sputum (previously treated with 3% sodium hydroxid as directed by Petroff) one of these prepared exactly according to the directions of Petroff and inspissated, the second a duplicate but autoclaved instead of inspissated, a third prepared by mixing one-fourth volume of neutral (to litmus) 20% glycerol broth of 4 times ordinary broth strength (prepared with sodium chlorid, Liebig's beef extract and Witte or Difco peptone) and 3 volumes of mixed white and yolk of egg to which finally is added 1: 10,000 gentian violet (Grübler) and inspissated, and a fourth duplicate of the third in ingredients but autoclaved.

The autoclaving is carried out by slanting the tubes in a horizontal self sealing autoclave (Duparquet, Huot and Moneuse Co.), rapidly expelling the air and replacing it by steam before the culture tubes have had time to warm up, shutting all valves and outlets quickly and maintaining a steam pressure of 10-15 lbs. for 30 minutes to 1 hour.

The growth of the tubercle bacilli obtained from 63 microscopically positive sputums by seeding a single tube of the 4 differently prepared mediums resulted as shown in Table 3.

A glance at Table 3 reveals that in cultivating tubercle bacilli on Petroff's medium practically the same results can be obtained regardless of whether the medium is prepared from fresh beef juice as recommended by Petroff or from Liebig's beef extract and peptone as carried out in the ordinary laboratory preparation of mediums. Autoclaving, which is a more rapid procedure, also does not possess any disadvantages as compared with inspissating of the egg medium. It may also be noted that no macroscopic cultures were obtained within 2 weeks' time.

TABLE 3

COMPARISON OF GROWTH OF TUBERCLE BACILLI FROM POSITIVE SPUTUMS ON MEDIUMS PREPARED ACCORDING TO PETROFF'S DIRECTIONS, INSPISSATED AND AUTOCLAVED, AND ON THE SAME MEDIUMS SUBSTITUTING BROTH PREPARED FROM LIEBIG'S BEEF EXTRACT AND PEPTONE FOR MEAT JUICE

Amount of Growth		Readings	Slight Macroscopic Growth†	Good Moderate Macroscopic Growth	Excellent Heavy Macroscopic Growth
Petroff's Egg Medium	Inspissated 43 Negative	4^{*}	1 1^{*} 1^{**}	4^{**}	1
		6	1 1^{**}	$1\text{-}3^{**}$	1
		8	4	1	
	Autoclaved 46 Negative	4	1 1^{*} 3^{**}	13^{**}	1
		6	2^{*}	2	1
		8	1	1	
Egg Medium Prepared with NaCl, Liebig's Beef Extract, and Peptone	Inspissated 46 Negative	4	0	1^{**}	
		6	3^{*} 2^{**}	$1\text{-}3^{**}$	1
		8	3	2	1
	Autoclaved 43 Negative	4	$1\text{-}2^{*}$ 1^{**}	3^{**}	1
		6	$1\text{-}1^{*}$ 3^{**}	$1\text{-}1^{**}$	2
		8	1	2	

Observations of the cultures were made at 2, 4, 6, and 8 weeks after inoculation. Since in no case was there a visible macroscopic growth after the 2 weeks, this interval is not included in the tabulation.

† A figure without an exponent indicates that the growth occurred as indicated on the chart but remained stationary throughout the 8 weeks observation period. A figure with an * as exponent indicates that the growth was noted as slight and became a moderately good growth. A figure with a ** as exponent indicates that the growth was noted as tabulated either as a slight or moderately good growth and became an excellent heavy growth.

These results were practically corroborated in an experiment in which 20 sets of tubes were seeded directly from 20 freshly isolated cultures of human tubercle bacilli.

It is also noteworthy that the scrupulous care recommended in preparing the eggs and meat juice is redundant, since not a single case of inherent contamination was obtained during the course of these investigations. Thorough hot air sterilization of all culture tubes, however, was a precaution observed. Before inoculation of the completed medium it was incubated (precautions being taken against drying) for at least 4 days to rule out contamination from this source.

In order to gain information on the efficiency of the routine cultivation of tubercle bacilli from the sputum by Petroff's method and also to obtain cultures of bacilli for other studies, 526 fresh sputums were cultivated using 2 tubes of Petroff's gentian violet medium for seeding each sputum. These sputums were all microscopically positive for tubercle bacilli after being stained by the ordinary carbolfuchsin method. From these 526 sputums there were obtained 144 positive macroscopic (27.3%) cultures of varying grades of intensity. The results are recorded in Table 4 from the standpoint of the individual sputum, in Table 5 from the standpoint of the individual culture tube, and in Table 6 from the standpoint of the number of bacilli found in the original stained sputum.

TABLE 4

RESULTS OF THE CULTIVATION OF TUBERCLE BACILLI FROM THE SPUTUM CONSIDERED FROM THE STANDPOINT OF THE INDIVIDUAL SPUTUM

Positive Cultures			Negative Cultures	Contaminated Cultures
+	++	+++		
22	47	75	100	282
Totals	144 (27.3%)		382	

If a sputum gave no growth in 1 tube and contamination in 1 tube, it was recorded under negative cultures.

If a sputum gave a growth in 1 tube and no growth or contamination in the second, it was recorded under the proper division of growth.

When growth occurred in 2 tubes from 1 sputum the best growth was recorded.

The conclusions to be drawn from Tables 4, 5 and 6 are briefly:

In cultivating tubercle bacilli from the sputum by Petroff's method but using only 2 culture tubes for seeding and macroscopic growth as criterion of positive findings there were obtained, from 526 sputums, 144 growths (27.3%). These results in no way vitiate the higher percentage obtained by other observers, notably Petroff, in smaller series in which more culture tubes were seeded, recultivation of contaminated cultures carried out, and microscopic growths considered. There was noted no relationship between the number of bacilli found in the original sputum examined microscopically after staining and the percentage positive growths obtained or the intensity of their growth.

In the continuation of cultures which have been isolated Petroff calls attention to the fact that the egg gentian violet medium is not always suitable for this purpose since perpetuation of the culture on this results in loss of power to grow with a simultaneous staining of the seeded material by means of the gentian violet. In order to note the effect of successive seeding of freshly isolated cultures of virulent human tubercle bacilli a series of 25 well grown cultures obtained from human sputum were transferred in each case to 2 fresh tubes of Petroff's gentian violet egg medium 2 successive times. The following results were obtained in these experiments.

TABLE 5

RESULTS OF THE CULTIVATION OF TUBERCLE BACILLI FROM THE SPUTUM CONSIDERED FROM THE STANDPOINT OF THE INDIVIDUAL CULTURE TUBE

	Positive Cultures			Negative Cultures	Contaminated Cultures
	+	++	+++		
	36	66	89	212	649
Totals	191 (18.1%)			861	

Of the 25 well grown cultures 9 gave good growths on both tubes seeded, 7 gave good growth on 1 tube and the other revealed a slight growth with 3 of these, 2 were contaminated (it is noteworthy that Petroff cultures which have been allowed to remain in the incubator, frequently develop contaminating growths even after good growths of tubercle bacilli have occurred and contaminations also occur on transplants which can only be attributed to the development of organisms from the original sputum which were merely held in abeyance by the sodium hydroxid treatment and 1:10,000 gentian violet; early transfers to fresh mediums reduce the possibility of development of these contaminators), and 2 revealed no growth; 2 gave slight growth in two tubes, 1 slight in one tube and negative in the other, and 6 were negative in both

TABLE 6

CLASSIFICATION OF CULTURES OBTAINED FROM POSITIVE SPUTUMS DEPENDENT ON THE NUMBER OF BACILLI FOUND IN THE ORIGINAL STAINED SPUTUM

	Growth							No Growth							Contaminations						
	Slide			Field				Slide			Field				Slide			Field			
	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100	1-5	6-25	Over 25	1-5	6-25	26-100	Over 100
	4	4	5	52	53	6	20	5	1	0	44	46	10	4	20	3	2	129	82	26	20
Totals	144							100							282						

If a sputum gave no growth in 1 tube and contamination in 1 tube, it was recorded under negative cultures.

If a sputum gave a growth in 1 tube and no growth or contamination in the second, it was recorded under growth.

tubes. Of 14 good growths which were seeded a second time, 5 gave good growths in two tubes, 3 in one tube and the other was contaminated in two instances and dried out in the third; 6 were negative, 2 of these contaminated in 2 tubes and 4 in one tube.

In the majority of the tubes in which growth failed to occur and no contaminations were found the original masses of tubercle bacilli seeded were stained with gentian violet, in this corroborating Petroff's findings.

SUMMARY

The routine cultivation of tubercle bacilli from microscopic positive sputum by Petroff's method, seeding 2 gentian violet egg medium tubes from each sputum, resulted in obtaining positive macroscopic cultures of varying grades on primary culture from 144 (27.3%) of 526 sputums. There was noted no relationship between the number of bacilli found in the original sputum and the percentage of positive growths obtained or their intensity.

The glycerol content or acidity (as pointed out by Szaboky, in experiments with egg medium) of the medium within wide ranges are of little importance in the determination of growth.

Egg medium prepared as directed by Petroff with fresh beef juice revealed no particular advantages over mediums in which was substituted sodium chlorid, beef extract, and peptone as used in ordinary broth. The inspissation of the Petroff gentian violet egg medium on 3 successive days also proved to have no advantages over a single autoclaving. Petroff's findings that the gentian violet egg medium is not suitable for the continued cultivation of tubercle bacilli was corroborated (24%, 6 of 25, failing to grow on 2 tubes in the first seeding and 43%, 6 of 14, failing to grow on 2 tubes in the second seeding).